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In re Application of:	:	
Lydie Poitout, et al.	:	Group: 1625
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Hedman and Costigan
1185 Avenue of the Americas
New York, NY 10036
July 23, 2008

LETTER

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Supplemental to the response of July 22, 2008, Applicants are submitting herewith copies of the Fan et al, Wessells et al, Vrinten et al and Alvaro et al references cited in the Thurieau declaration.

Respectfully submitted,

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CAM:mlp
Enclosures

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Marie-Louise Pinset

basic conclusion, that $[Ca^{2+}]$ transients peak close to the soma with little influx in the distal braches of layer 1, would thus not be affected. In some cases (Fig. 4*b*, *d*), to dissipate dye gradients, Ca^{2+} transient were recorded more than 30 min after electrode withdrawal. All data are presented as mean ± s.e.m.

Brain-slice preparation, imaging and electrophysiology. Rats (4–6 weeks old) were deeply anaesthetized (50 mg ml^{-1} Nembutal) and decapitated. The brain was removed and coronal slices (300 μm thick) from somatosensory cortex were cut with a vibratome. Slices were kept at 33 °C for 30 min and subsequently stored at room temperature (22–24 °C). After 1–5 h slices were transferred to a submerged recording chamber (22–24 °C). The artificial cerebrospinal fluid contained (in mM): 124 NaCl, 26 $NaHCO_3$, 3 KCl, 1.25 NaH_2PO4, 2 $MgSO_4$, 10 dextrose, 2 $CaCl_2$, saturated with 95% O_2/5% CO_2 (pH 7.3; osmolarity, 290–300 mOsm). In some cases 1 μM of tetrodotoxin was added. Whole-cell recordings were established under visual control, and recordings were made with a patch-clamp amplifier (EPC-7; List Electronics) operating in current-clamp mode. Resistance compensation was performed off-line. Electrodes had resistances of 7–10 MΩ when filled with internal solution containing (in mM): 135 K-methanesulphonate (Fluka), 10 K-HEPES, 2 $MgCl_2$, 3 Na_2ATP, 0.2 EGTA and 0.1 calcium green-1 (Molecular Probes) (pH, 7.2–7.3; osmolarity, 280 mOsm). In some experiments 6 mM QX-314 was added. Access resistances after break-in were 15–30 MΩ. Measurements were started 20 min after break-in.

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Role of melanocortinergic neurons in feeding and the *agouti* obesity syndrome

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DOMINANT alleles at the *agouti* locus (*A*) cause an obesity syndrome in the mouse, as a consequence of ectopic expression of the *agouti* peptide[1–6]. This peptide, normally only found in the skin, is a high-affinity antagonist of the melanocyte-stimulating hormone receptor (MC1-R)[7], thus explaining the inhibitory effect of *agouti* on eumelanin pigment synthesis. The *agouti* peptide is also an antagonist of the hypothalamic melanocortin-4 receptor (MC4-R)[7–9]. To test the hypothesis that *agouti* causes obesity by antagonism of hypothalamic melanocortin receptors[7], we identified cyclic melanocortin analogues[10] that are potent agonists or antagonists of the neural MC3 (refs 11, 12) and MC4 receptors. Intracerebroventricular administration of the agonist, MTII, inhibited feeding in four models of hyperphagia: fasted C57BL/6J, *ob/ob*, and A^y mice, and mice injected with neuropeptide Y. Co-administration of the specific melanocortin antagonist and *agouti*-mimetic SHU9119 completely blocked this inhibition. Furthermore, administration of SHU9119 significantly enhanced nocturnal feeding, or feeding stimulated by a prior fast. Our data show that melanocortinergic neurons exert a tonic inhibition of feeding behaviour. Chronic disruption of this inhibitory signal is a likely explanation of the *agouti* obesity syndrome.

The *agouti* peptide acts as a paracrine factor in the regulation of eumelanin (brown–black pigment) synthesis, and in the induction of obesity[13]. The non-endocrine nature of the peptide and the lack of homogeneous preparations have complicated analysis of the activities of the peptide *in vivo*. Screening analogues of the cyclic lactam melanocortin agonist MTII (Ac-Nle^4-c[Asp^5, D-Phe^7, Lys^{10}]α-MSH-(4-10)-NH_2) led to the identification of the *agouti*-mimetic SHU9119 (Ac-Nle^4-c[Asp^5, D-2′Nal^7, Lys^{10}]α-MSH-4-10)-NH_2)[10] (Fig. 1*a*). This compound shares pharmacological properties with *agouti* peptide in that it is a potent antagonist of MC4-R and a less potent antagonist of the MC3-R[7] (Fig. 1*b*). The closely related analogue MTII (ref. 14) is identical to SHU9119 with the exception of a D-phenylalanine in place of the D-2′naphthylalanine, and is a full agonist of the MC3-R and MC4-R (Fig. 1*b*).

Mice were induced to feed by food deprivation for 16 h before intracerebroventricular (ICV) administration of the nonspecific melanocortin agonist MTII. In comparison to vehicle-injected animals, MTII was found to produce a potent inhibition of feeding within one hour of administration (Fig. 2*a*). At the highest dose (3 nmol), food intake was significantly inhibited for up to 4 h after administration ($P < 0.001$), and decreased food intake continued for the next 4 h, with normal rates resuming about 8 h after treatment. There was a dose–response relationship between MTII dose and feeding inhibition, with an IC_{50} at the 2-h time point of 0.6 nmol. Inhibition of feeding with 3 nmol MTII was blocked by co-administration of 6 nmol SHU9119 (Fig. 2*b*; $P < 0.001$), demonstrating that the effect results specifically from agonist binding to MC4-R and/or MC3-R.

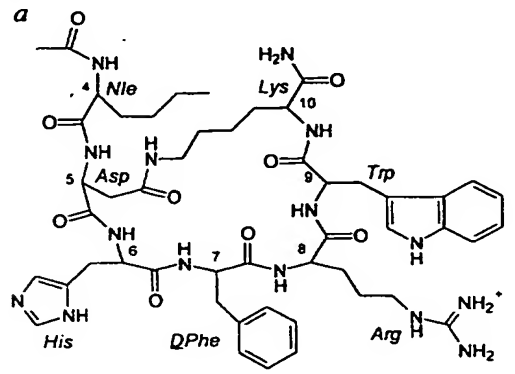
MTII-treated mice were alert and exhibited no unusual behaviour relative to controls. The effect of MTII on locomotor activity was tested by using sound- and light-proof cages containing multiple light-beam detectors. The movements of MTII- and vehicle-treated mice were measured during two 90-min time periods (0.5–2 h, Fig. 2*c*; 2.5–4 h, data not shown) after ICV administration. The higher initial activity, indicative of exploratory behaviour, and continued locomotion were indistinguishable between the two groups, indicating that the inhibition of feeding was not due to decreased arousal or locomotion. In contrast to its long-term inhibition of feeding (4–8 h), MTII only inhibited drinking in water-deprived animals during a brief time period (<1 h) after administration (Fig. 2*d*).

The administration of MTII also inhibited food intake in three other models of hyperphagia, involving C57BL/6J-*Lep*^{ob}, C57BL/6J-A^y and neuropeptide Y (NPY)-injected C57BL/6J mice. The hyperphagia in these models can be seen clearly by comparing the 12 h food intake after a fast in vehicle-injected C57BL/6J (2.4 g; Fig. 2*a*), C57BL/6J-A^y (3.7 g, Fig. 3*a*) and C57BL/6J-*Lep*^{ob} (3.7 g; Fig. 3*c*) animals. As expected, MTII treatment inhibited food intake after a 16-h fast in the C57BL/6J-A^y mouse (Fig. 3*a*; $P < 0.05$).

MTII, when co-administered with NPY, significantly inhibited the profound stimulation of feeding normally induced by NPY,

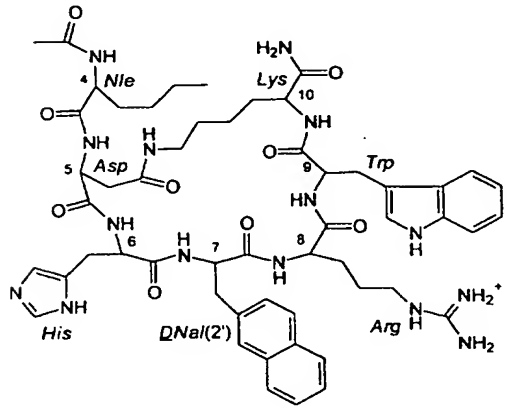
measured over a 3-h period (Fig. 3*b*; $P < 0.005$). Co-administration of an approximately twofold molar excess of MTII produced a 74% inhibition of NPY-stimulated food intake at the 3-h time point. Finally, MTII very potently inhibited hyperphagia caused by the absence of leptin in the C57BL/6J-*Lep*^{ob} mouse (Fig. 3*c*; $P < 0.001$). The C57BL/6J-*Lep*^{ob} mouse was also used to test the ability of MTII to regulate feeding after intraperitoneal administration. Moderate doses (100 nmol) of MTII inhibited feeding in the C57BL/6J-*Lep*^{ob} mouse (Fig. 3*d*; $P < 0.001$), but low doses (10 nmol) did not (data not shown). The kinetics were similar to those seen with ICV administration, with a potent inhibition of

a



Ac-Nle^4-Asp^5-His^6-DPhe^7-Arg^8-Trp^9-Lys^{10}-NH$_2$

MT-II



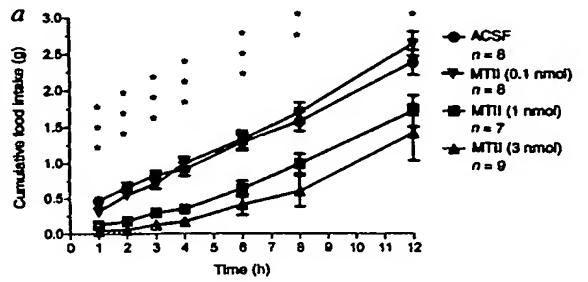
Ac-Nle^4-Asp^5-His^6-DNal(2')^7-Arg^8-Trp^9-Lys^{10}-NH$_2$

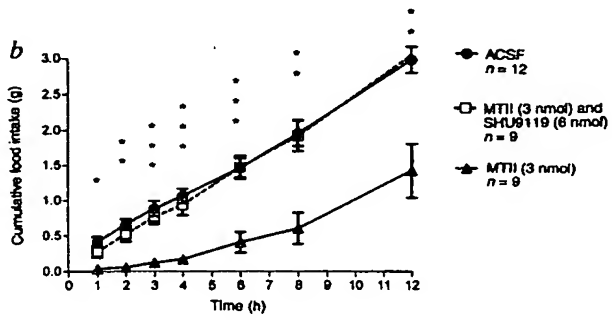
SHU-9119

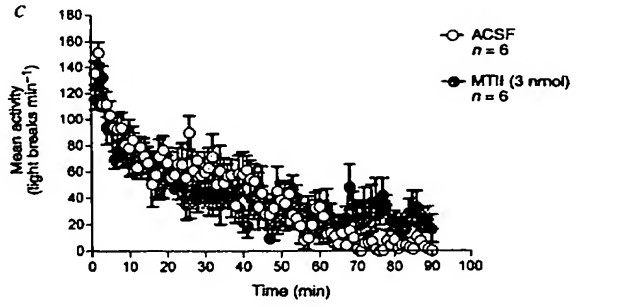
b

	rat MC3-R	mouse MC4-R
agouti	antagonist IC_{50}>100 nM	antagonist IC_{50}=3.9 ±0.6 nM
SHU9119	antagonist IC_{50}=4.5 ±2.1 nM	antagonist IC_{50}=0.36 ±0.13 nM
MTII	agonist EC_{50}= 0.27 ±0.23 nM	agonist EC_{50}= 0.057 ±0.024 nM

FIG. 1 Structure and properties of the cyclic lactam melanocortin compounds MTII and SHU9119. *a*, Chemical structures. *b*, Pharmacological properties of MTII and SHU9119 compared with murine *agouti* peptide. IC_{50} values represent ligand concentration required for half-maximal inhibition of binding of ^{125}I-[Nle^4, D-Phe^7]α-MSH tracer. EC_{50} values represent ligand concentration required for half-maximal activation of a cAMP-responsive β-galactosidase reporter gene. Specific competition of [Nle^4, D-Phe^7]α-MSH binding to the rat MC3-R by 100 nM *agouti* peptide was observed, but accurate IC_{50} values could not be determined in the absence of a peptide preparation of higher concentration and purity.







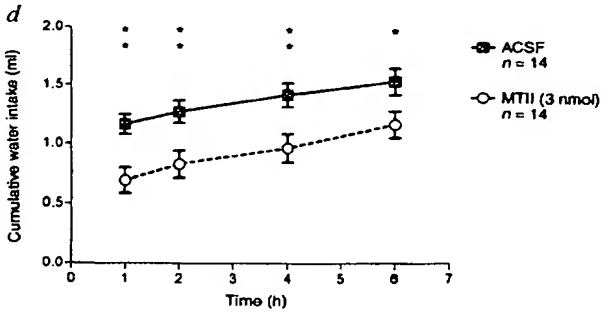


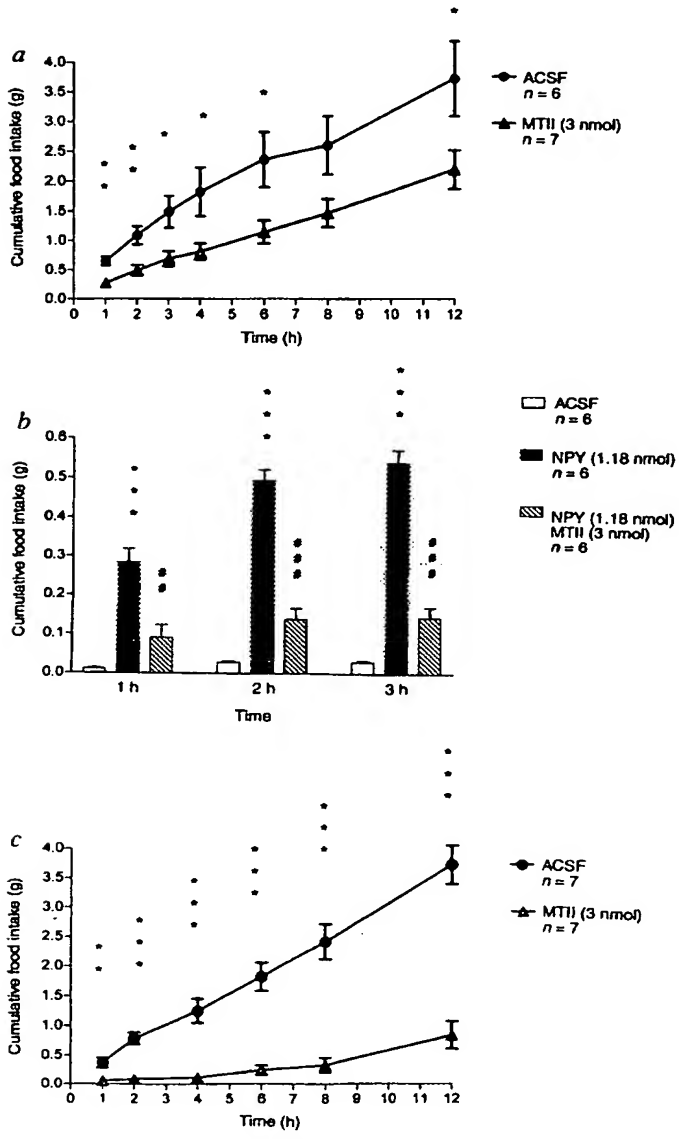
FIG. 2 Inhibition of feeding by ICV administration of the melanocortin agonist MTII. The melanocortin agonist MTII produced a specific and dose-responsive inhibition of feeding in male C57BL/6J mice induced to eat by 16 h food deprivation without altering locomotor activity and with only short-term effects on drinking behaviour. *a*, Inhibition of feeding by MTII is dose responsive. *b*, SHU9119 (6 nmol) specifically blocks MTII inhibition of feeding. *c*, ICV administration of MTII does not alter locomotor activity. *d*, MTII does not produce long-term inhibition of drinking. Significance values indicated for individual time points are: *a*, 3 nmol MTII versus ACSF; *b*, 3 nmol MTII versus 3 nmol MTII + 6 nmol SHU9119. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

feeding for the first 4 h. Melanocortins may also affect metabolism directly because an acute effect of melanocortin agonists on serum insulin could also be seen in the severely hyperinsulinaemic C57BL/6J-*Lep*^{ob} mouse (Fig. 3*e*), and to a lesser extent in the mildly hyperinsulinaemic C57BL/6J-*A*^{Y} or normal C57BL/6J mouse (data not shown). Two hours after intraperitoneal administration of [Nle^4, D-Phe^7]α-MSH (NDP-MSH), a linear analogue of MTII that has similar pharmacological properties[10], serum insulin levels were 58% lower than in saline-injected controls ($P < 0.001$), and SHU9119 partly blocked the effect ($P < 0.01$); similar but less pronounced effects were seen with MTII.

Daytime food intake in animals fed *ad libitum* was not stimulated by ICV administration of 6 nmol SHU9119 (data not shown). However, SHU9119 induced a significant increase in food intake when administered to animals before lights out (Fig. 4*a*; $P < 0.02$ at 2 h and 4 h). In this model, 3 nmol SHU9119 led to a mean 29% increase in cumulative food intake at 4 h and a 53% increase in the first 2 h. As anticipated, MTII also significantly inhibited normal nocturnal food intake. Hyperphagia was also induced following SHU9119 treatment of fasted animals (Fig. 4*b*, $P < 0.001$). Stimulation of feeding was evident at approximately 2 h post-treatment, and continued for 12 h to produce a mean 34% increase in food intake relative to vehicle-injected controls.

Our results demonstrate that a melanocortin agonist can inhibit feeding potently in four models of hyperphagia, and also inhibit normal nocturnal food intake. Furthermore, an MC3-R/MC4-R antagonist specifically induced hyperphagia, demonstrating that melanocortinergic neurons, probably from the arcuate nucleus[15,16], exert a tonic inhibitory effect on feeding behaviour. In a related study, a knockout mouse strain lacking MC4-R has been demonstrated to recapitulate several aspects of the *agouti* obesity syndrome, including hyperphagia, hyperinsulinaemia, late-onset obesity, and increased linear growth[17]. Taken together, these results indicate that obesity in the C57BL/6J-*A*^{Y} mouse results from the cumulative effect of chronic antagonism of MC4-R signalling in the brain by *agouti* peptide. Although it is known that pro-opiomelancortin mRNA levels in the arcuate nucleus are regulated by metabolic state[18–20], the relevant physiological inputs to these neurons, and downstream mechanism by which they regulate feeding and possibly ingestive behaviour in general, remain to be determined.

The elevation of NPY expression in the arcuate nucleus in the C57BL/6J-*Lep*^{ob} mouse[21] is one downstream factor signalling the induction of hyperphagia in the absence of leptin[22]; leptin has been shown to bind to arcuate nucleus neurons and downregulate NPY expression there[23,24]. No change in arcuate nucleus expres-



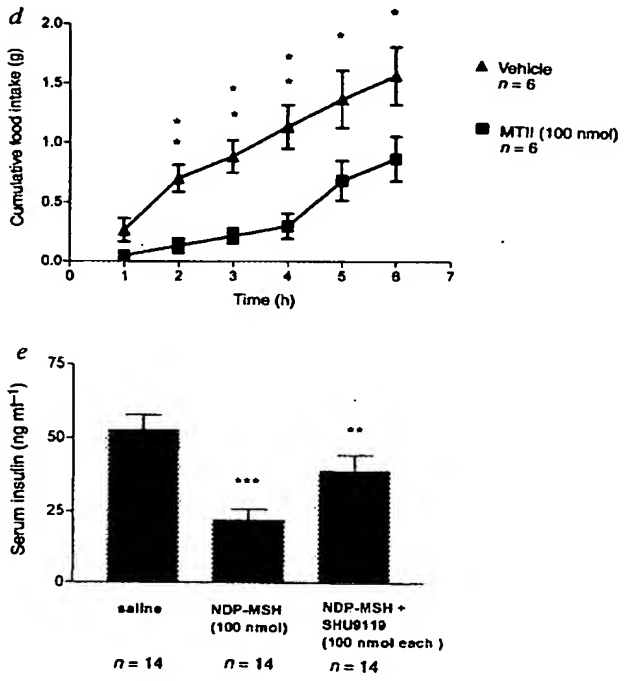


FIG. 3 Melanocortin agonist MTII inhibits feeding in C57BL/6J-*A*^{Y}, C57BL/6J-*Lep*^{ob}, and NPY-treated C57BL/6J mice. *a*, Inhibition of feeding by ICV administration of MTII in C57BL/6J-*A*^{Y} mice (females, 19–28 g). *b*, Inhibition of feeding by ICV administration of MTII in C57BL/6J mice (females, 21–25 g) stimulated to feed by co-administration of NPY. *c*, Inhibition of feeding by ICV administration of MTII in C57BL/6J-*Lep*^{ob} mice (females, 48–69 g). *d*, Inhibition of feeding in C57BL/6J-*Lep*^{ob} mice by intraperitoneal administration of MTII (females, 40–45 g). *e*, Effect of intraperitoneal administration of the melanocortin agonist [Nle^4, D-Phe^7]α-MSH on serum insulin in C57BL/6J-*Lep*^{ob} mice (females, 45–60 g). Mice were fasted overnight as described, with the exception of the NPY-treated animals, which were fed *ad libitum*. Animals treated intraperitoneally received 100 nmol of each compound indicated, or an equal volume (100 μl) of saline alone. Significance indicated for individual time points: *a*, 3 nmol MTII versus ACSF; *b*, * 1.18 nmol NPY versus ACSF; # 1.18 nmol NPY versus 1.18 nmol NPY + 3 nmol MTII; *c*, 3 nmol MTII versus ACSF; *d*, 100 nmol MTII versus saline; *e*, 100 nmol NDP-MSH versus saline, 100 nmol NDP-MSH versus 100 nmol each of NDP-MSH + SHU9119.

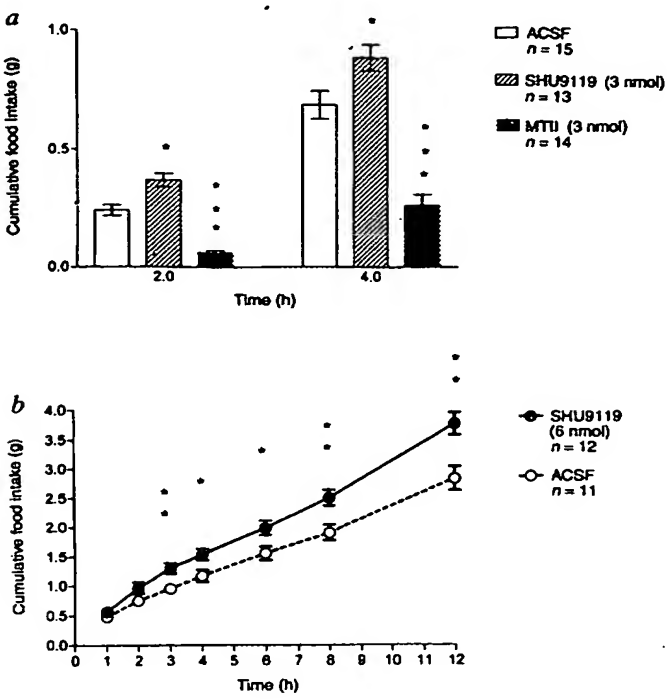


FIG. 4 Stimulation of feeding by ICV administration of the melanocortin antagonist SHU9119. Data show cumulative food intake as a function of time after administration of the compounds shown. *a*, Stimulation of nocturnal feeding with SHU9199 in C57BL/6J mice fed *ad libitum*. *b*, Stimulation of daytime feeding by SHU9119 administration in fasted C57BL/6J mice. Male C57BL/6J mice were maintained on a normal 12 h/12 h light/dark cycle with food (Purina mouse chow) and water *ad libitum*, and treated just before lights off (*a*). In *b*, mice were fasted for 16 h before treatment. Asterisks indicate significance of drug versus control ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.01$).

sion of NPY mRNA was seen in C57Bl/6J-A^y mice. However, NPY is induced in the dorsomedial hypothalamic nucleus in obese C57BL/6J-A^y and MC4-R knockout mice, which also identifies NPY as a potential downstream effector in these two melanocortinergic obesity models[25]. □

Methods

Peptide preparation and analysis. SHU9119 and MTII were synthesized and purified to homogeneity[10,14]. Murine *agouti* peptide was produced in the baculovirus system, as ref. 7 but with one modification: *agouti* was purified from baculovirus supernatants by 0.6-M NaCl step elution from an EconoS cation exchange column (BioRad); peptide was approximately 60% pure. Agonist and antagonist activities of MTII, SHU9119 and *agouti* were first ascertained by examining their ability to stimulate the adenylyl cyclase signalling pathway in HEK293 cell lines expressing the indicated receptor (rat MC3-R and mouse MC4-R), or to inhibit stimulation by α-MSH. A direct adenylyl cyclase assay[26] was used to analyse *agouti*, but MTII and SHU9119 were analysed using a cAMP-responsive β-galactosidase reporter construct[27] to detect intracellular cAMP levels. Competition binding experiments were performed[10], with nonspecific binding determined as the amount of radioactivity bound in the presence of 5×10^{-6}M cold [Nle^4, D-Phe^7]α-MSH, and was typically 3–5% of total counts bound. Values shown are the mean and standard deviations from 2–3 determinations.

Injections and feeding assays. Mice (20–30 g male C57BL/6J, unless indicated otherwise) were maintained on a normal 12 h/12 h light/dark cycle with food (Purina mouse chow) and water *ad libitum*. Animals were housed individually for 24 h, distributed into weight-matched experimental and control groups, and injected with vehicle or vehicle plus drug as indicated. Fasting consisted of food deprivation from 18:00 to 10:30 to stimulate feeding during the daytime experimental period. Animals were lightly anaesthetized with halothane, and administered buffered artificial cerebrospinal fluid (ACSF, in mM) (137.9 NaCl, 3.37 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 1.45 $NaH_2PO_4 \cdot H_2O$, 4.85 $Na_2HPO_4 \cdot 7H_2O$, pH 7.4) as vehicle, or compounds indicated in a volume of 2 μl ACSF into one lateral ventricle. Freehand ICV injections were performed essentially as described[28]. A high efficiency of injection into the lateral ventricle was achieved, as monitored using methylene blue, in training sessions before the experiments were started. The compounds indicated, in a 2 μl volume of ACSF, were administered slowly over approximately 15 s, and the needle removed after 35 s. Mice were allowed to recover from anaesthesia and placed into a cage containing a premeasured quantity of food pellets in a spill-free cup. Only one moribund animal and no fatalities resulted from the 206 animals receiving ICV injections in this study; this animal and its matched pair were excluded. Food remaining was briefly removed and weighed at the time intervals indicated. Data points indicate the mean, bars indicate standard error. Significance of effects over time was determined by analysis of variance (ANOVA) with repeated measures. Significance of drug effects at individual time points was determined by two-way ANOVA, and is indicated in each figure ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

Locomotor assay. Locomotor assays were performed by first injecting 3 nmol MTII or ACSF into mice, 3 h after lights on, with mice on food and water *ad libitum*. At 0.5 h after the injection, 12 mice were placed without prior habituation into separate boxes containing multiple infrared light sources and photodetectors. Locomotor activity was then monitored for two 90-min periods, 0.5–2 and 2.5–4 h after injection. The boxes were contained within separate ventilated light- and sound-attenuating chambers (Coulbourn model E10-20). Disruption of the infrared beams, with 10 ms resolution, was tallied independently for each 1-min time period in each cage. Data points indicate the mean total activity (number of light breaks) for 6 animals in each experimental group, and bars indicate standard error. Four-way ANOVA was used to analyse the data, and indicated that there was no significant effect of drug on locomotor activity.

Drinking assay. Mice on food *ad libitum* were deprived of water for 24 h before the experiment. They were then injected ICV with ACSF or MTII (3 nmol) and housed individually in cages containing volumetric water bottles but no food. Water consumption was measured hourly.

Insulin assay. C57BL/6J-*Lep*^{ob} mice (female, 45–60 g) were injected intraperitoneally 1 h after lights on with 100 μl saline, 100 nmol NDP-MSH, or 100 nmol of each NDP-MSH + SHU9119. After 2.5 h, 50 μl blood was withdrawn by tail bleed, and the serum separated and assayed (10 μl) for insulin, in duplicate, by radioimmunoassay (Linco).

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Melanocortin receptor agonists, penile erection, and sexual motivation: human studies with Melanotan II

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We review our experience with Melanotan II, a non-selective melanocortin receptor agonist, in human subjects with erectile dysfunction (ED). Melanotan II was administered to 20 men with psychogenic and organic ED using a double-blind placebo-controlled crossover design. Penile rigidity was monitored for 6 h using RigiScan. Level of sexual desire and side effects were reported with a questionnaire.

In the absence of sexual stimulation, Melanotan II led to penile erection in 17 of 20 men. Subjects experienced a mean of 41 min Rigiscan tip rigidity > 80%. Increased sexual desire was reported after 13/19 (68%) doses of Melanotan II *vs* 4/21 (19%) of placebo ($P < 0.01$). Nausea and yawning were frequently reported side effects due to Melanotan II; at a dose of 0.025 mg/kg, 12.9% of subjects had severe nausea.

We conclude that Melanotan II is a potent initiator of penile erection in men with erectile dysfunction. Our findings warrant further investigation of melanocortin agonists and antagonists on penile erection. *International Journal of Impotence Research* (2000) **12**, Suppl 4, S74–S79.

Keywords: penile erection; MSH; sex behavior; impotence

Introduction

Alpha-melanocyte-stimulating hormone (α-MSH) and adrenocorticotropin (ACTH), known as the melanocortins, have been implicated in the control of penile erection and sex behavior in animals.[1–5] Five melanocortin receptor (MC-R) subtypes have been cloned, and reveal different functions based on localization.[6] MC1-R and MC2-R are the classical melanocytic and adrenocortical receptors in the skin and adrenal cortex respectively.[7,8] MC3-R is principally present in the brain, with low levels of expression in the gut.[9] MC4-R is restricted to the nervous system; blockade of this receptor partially abolishes αMSH-induced erection in rats.[5] MC5-R has been localized in exocrine and endocrine glands including the reproductive tract of the rat.[6,7]

Melanocortins influence important homeostatic behaviors mediated by the hypothalamus. Melanocortinergic neurons exert a tonic inhibition of feeding behavior. Chronic disruption of this inhibitory signal by the agouti peptide is a likely explanation of the agouti obesity syndrome in mice.[10] The agouti peptide is an MCR-4 antagonist. Melanocortins regulate sexual behavior including penile erection, sexual motivation, and, in the female rat the secretion of sexual attractants from the preputial gland.[6] α-MSH and ACTH are believed to act downstream from dopamine and oxytocin in the hypothalamic proerectile centers adjacent to the third ventricle.[2] MC5-R expression among peripheral tissues provides a functional coherence between central and peripheral control of sex behavior.[6]

A number of melanocortin agonists have been synthesized to enhance skin pigmentation.[11] Melanotan I, a linear peptide analog of α-MSH, causes tanning.[12] Melanotan II, a cyclic non-selective melanocortin receptor agonist, initiates erections in rats, dogs, and humans.[13,14] We report our experience with Melanotan II in human subjects with erectile dysfunction (ED).[13,15]

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Materials and methods

Subjects

Men aged 18–75 y with a chief complaint of ED were enrolled in two studies.[13,15] In Study 1 men with no organic etiologies and normal nocturnal penile tumescence (> 10 min of tip rigidity > 70%)

were enrolled and designated as having psychogenic erectile dysfunction. Men with ED and major organic risk factors were enrolled in Study 2. The pertinent characteristics of the study populations are listed in Table 1. Erectile dysfunction was defined as the persistent inability to obtain and maintain an erection sufficient for sexual satisfaction.[16] The Human Subjects Committee of the University of Arizona approved the studies, and written informed consent was obtained on all subjects.

Peptide chemistry:

Melanotan II is a synthetic cyclic heptapeptide, Ac-Nle-c[Asp-His-DPhe-Arg-Trp-Lys]-NH_2, which contains the 4–10 melanocortin receptor binding region common to ACTH and MSH, but with a lactam bridge and four amino acid substitutions (Figure 1).

Table 1 Comparison of patient data in two studies (mean values)

Variable	*Study 1 (psychogenic)*	*Study 2 (organic)*
n	10	10
Age (y)	47.4	56.2
Testosterone (mg/ml)	450	362
Pre study NPT events	2.9	2.7
NPT tip rigidity > 70%(min)	47.4	9.3
Organic risk factors	0	2.2

Drug synthesis and purification were carried out as previously described.[17]

Experimental design

A double blind, placebo-controlled crossover design was used. Melanotan II (0.025–0.157 mg/kg) and vehicle were each administered by the investigator twice by subcutaneous injection for a total of four injections; study drug doses were separated by at least 48 h. The order of administration was randomly assigned.

Penile erection was measured with real-time RigiScan monitoring in the home situation (Figure 2). Subjects were instructed to avoid erotic stimuli and to remain awake for the 6-h session. Subjects recorded the number and duration of erectile events. In Study 2, sexual desire was scored from 1 to 5 using a modification of IIEF question 12.[18] Side effects (none, mild, moderate, or severe) were recorded for: nausea; yawning or stretching; facial flushing; decreased appetite; increased appetite; and drowsiness. Subjects denoted onset of symptoms, duration, and measures taken to relieve them. Antiemetics were not prescribed.

Statistical analysis

Mean values of RigiScan parameters after Melanotan II and placebo were compared with the one-tailed

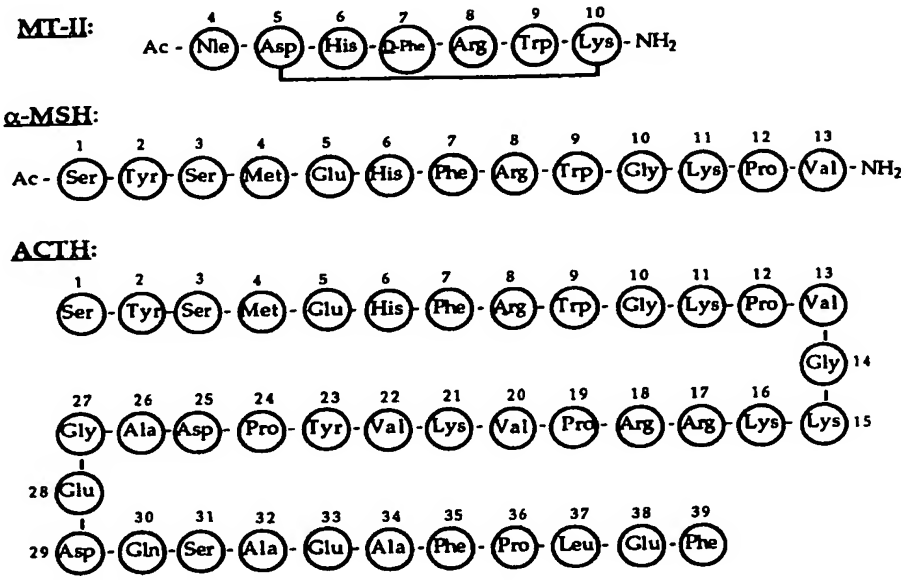

Figure 1 Structure of Melanotan II, α-MSH, and ACTH. Reprinted with permission.[14]

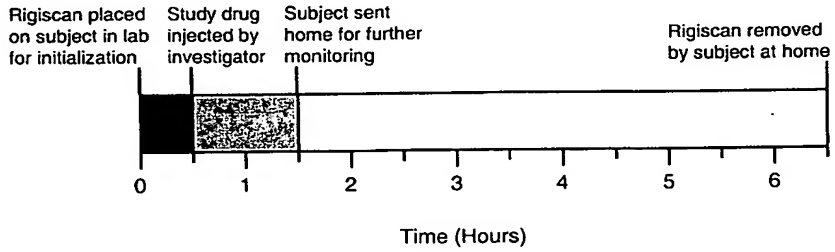

Figure 2 Schema of the experimental protocol. Reprinted with permission.[13]

Student's *t*-test. Side effects due to Melanotan II and placebo were compared with Fisher's exact test.

Results

Twenty subjects aged 22–67 y (mean 51.6 y) were enrolled and completed the studies between July 1995 and December 1998.[13,15] Thirty-nine total injections of Melanotan II were given and 41 of placebo (one subject received three placebo injections and one of Melanotan II due to erroneous administration of vials discovered after completion of the study). No patient withdrew because of side effects.

Of 20 subjects, 17 reported subjectively apparent erection on at least one of two injections of Melanotan II. Overall, erectile activity was reported with 27/39 (69%) Melanotan II injections and 1/41

Table 2 Real-time RigiScan activity after Melanotan II (MT II) and placebo (mean values)

	Psychogenic study		*Organic study*	
RigiScan parameter	*MT II Study 1*	*Placebo Study 1*	*MT II Study 2*[a]	*Placebo Study 2*
% of injections with response	75	0	63	4.7
Erectile events (no.)	3.45	2.35	2.66	0.7
Erectile latency (min)	127.5	NA	97.8	NA
Total erectile duration (min)	163.4	54.5	97.5	25.3
Tip rigidity 80–100%(min)	38.0	3.0	45.3	1.9
Tip rigidity 60–79%(min)	40.3	4.5	10.1	1.1
Tip rigidity activity units	78.4	9.8	58.6	4.54
Tip tumescence activity units	49.7	13.6	29.1	6.0

[a]RigiScan data based on 16 injections due to loss of data from three injections in three subjects, one of whom who reported no erection and two of whom had significant erections (60 and 90 min duration, 9/10 rigidity by visual analog scale).[15]

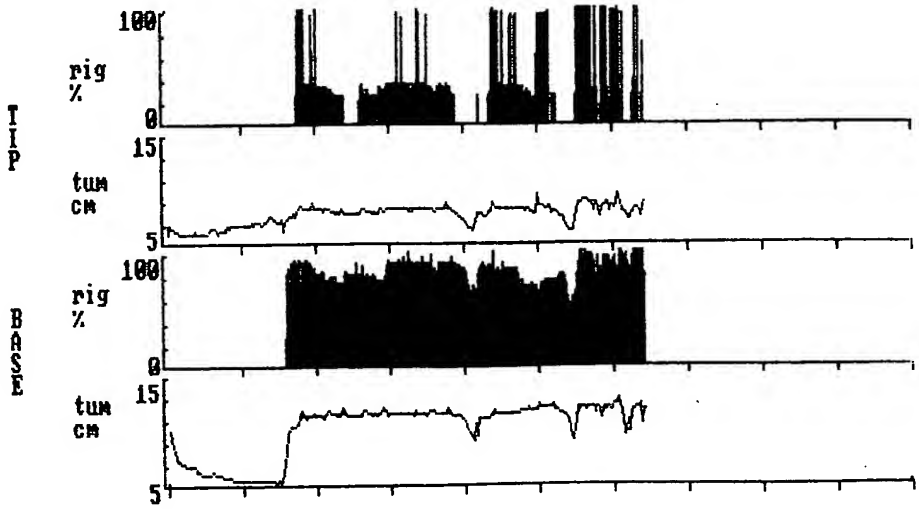

Figure 3 RigiScan tracing of a subject with organic ED who experienced penile erections after 0.025 mg/kg Melanotan II. Note the intervals without significant tip rigidity.

placebo injections ($P < 0.01$). Twelve subjects responded to each Melanotan II injection, five responded to only one of two doses, and three men had no erectile activity with Melanotan II.

RigiScan results showed statistically significant differences in erectile activity between Melanotan II and placebo (Table 2). Latency to first erection ranged from 15 to 270 min (mean 115). Duration of rigidity between 80 and 100% ranged from 0 to 254 min on Melanotan II (mean 41.0). Tip Rigidity Activity Units were 78.4 and 58.6 in psychogenic and organic ED patients respectively ($P = 0.49$). The subject with 254 min of rigidity had two episodes of complete detumescence dividing the erections (see Figure 3), and no subject reported a painful erection.

Heightened sexual desire was reported in Study 2 after 13/19 (68%) doses of Melanotan II *vs* 4/21 (19%) of placebo ($P = 0.0034$). Table 3 shows the individual responses. Of the 10 subjects reporting a moderate or high level of desire, all but one developed a penile erection. Mean level of sexual desire (scale 1–5) was 2.47 after Melanotan II *vs* 1.24 on placebo ($P < 0.001$).

Nausea and stretching/yawning occurred significantly more frequently with Melanotan II than placebo (Table 4), but no serious or unexpected adverse events occurred. Nausea was reported with 15/39 (38%) injections of Melanotan II, including severe nausea in six cases (15.3%, $P < 0.05$ *vs* placebo). One subject vomited in association with an episode of severe nausea. Four of 31 (12.9%) injections of 0.025 mg/kg Melanotan II led to severe nausea. No subject experienced severe nausea with both administrations of Melanotan II, and symptoms were reduced, in eight of 11 instances, on second dosage of the drug.

Table 3 Effect of Melanotan II on sexual desire[a]

Sexual desire[b]	*MT II* $n = 19$	*Placebo* $n = 21$
Very low or none at all	6	17
Low	3	3
Moderate	5	1
High	5	0
Very high	0	0

[a]Data from Study 2 only.[15]
[b]Modified Question 12 of IIEF.[18]

Table 4 Clinically important side effects of Melanotan II and placebo

Side effect	*Melanotan II* *(n = 39)*	*Placebo* *(n = 41)*
Nausea		
None	23	37
Mild	8	3
Moderate	1	0
Severe	6[a]	1
Stretch/yawn		
None	17	36
Mild	14	3
Moderate	5	2
Severe	3	0

[a]Two of these six subjects received > 0.1 mg/kg Melanotan II.

Discussion

Melanotan II, a non-specific MCR agonist, initiates erections in men with organic and psychogenic erectile dysfunction. Seventeen of 20 subjects reported penile erection, although not with each administration. The majority of erections induced by Melanotan would be considered sufficient for sexual intercourse: tip rigidity time > 80% averaged 41 min. The magnitude and duration of erectile activity was greater in the psychogenic ED patients than organic ED patients, although this difference was not statistically significant. The nature and severity of the erectile dysfunction was not completely characterized in these studies, making comparison of results between the two groups difficult. We found no significant correlation between duration of prestudy NPT and response to Melanotan II, although subjects only underwent one night of NPT testing.

The mean erectile latency time of 112.6 min for Melanotan II is long for a clinically useful drug. However, the addition of erotic stimulation may lead to responses that are more rapid. It is possible that the RigiScan device, through intermittent constriction of the penis, provides some sexual stimulation. Melanotan II in all likelihood must cross the blood brain barrier for its activity, which may explain the prolonged time to peak effect. Modification of drug delivery may enhance acceptability for clinical use. The time at which nausea was first reported, on average 168 min after injection, suggests that the gastrointestinal side effects are centrally mediated as well. The exact locus of action of Melanotan II remains unknown, but strong circumstantial evidence points to a central mechanism. Intracerebroventricular administration of Melanotan II in rats leads to erection and yawning, and no change in intracavernous pressure was observed after intracavernous injection (unpublished data). Melanotan I, a non-cyclic MCR agonist that does not cross the blood brain barrier, has no erectogenic activity. Further animal studies using selective melanocortin antagonists and agonists and receptor localization will be necessary to identify its pharmacological mechanism.

We could not differentiate Melanotan II responders from non-responders based on NPT criteria, testosterone levels, or etiology of ED. Our combined data make it unlikely that Melanotan II could be used as a diagnostic tool to differentiate organic and psychogenic etiologies. Differences were noted in the onset and duration of erectile activity between

psychogenic and organic subjects. In the absence of pharmacokinetic data on these subjects, any explanation of these differences remains speculative. It is intriguing to consider, however, that psychogenic inhibition of arousal may contribute to a delayed onset of erectile activity, whereas organic etiologies reduce the total duration and magnitude of response.

The increased sexual desire scores after Melanotan II may reflect the wording of IIEF question 12, which describes sexual desire as 'wanting to have a sexual experience, thinking about having sex, or feeling frustrated due to lack of sex'.[18] These responses certainly would not be surprising in men with ED who develop penile erections and cannot engage in sexual activity. Investigation of this finding in subsequent home use studies would be essential before further commercialization of the drug.

The safety profile of Melanotan II is acceptable: no serious adverse event occurred, and only one subject requested antiemetic therapy. Nausea and stretching/yawning were reported more commonly after Melanotan II than placebo in both studies. While 38% of subjects reported nausea, the incidence of severe nausea at our preferred dose of 0.025 mg/kg was 12.9%. We noted a reduction in the incidence and severity of nausea with the second administration of the drug, suggesting a first dose effect. Only one subject reported skin pigmentation, and a cumulative dose of 0.1 mg/kg is necessary for skin tanning.[14]

Yawning occurred with a significantly higher frequency after Melanotan II than placebo. We believe that this phenomenon is analogous to the stretching/yawning syndrome observed in rats. The stretching/yawning syndrome, an ancestral vestige that subserves arousal, is mediated by hypothalamic centers in proximity to the paraventricular nucleus.[2]

Melanotan II may eventually prove to be beneficial for patients with ED. However, the incidence of severe nausea and prolonged latency time raise questions about the potential clinical applications of the drug.[15] The pharmacokinetics and lowest effective dose of Melanotan II must be determined in order to reduce side effects without reduction in erectogenic activity.

Conclusions

Melanotan II, a non-selective MCR agonist, initiated penile erections in 69% of administrations in men with psychogenic and organic ED. Mean duration of real-time RigiScan tip rigidity > 80% was 41 min; the time to onset (mean 112 min) was longer for men with psychogenic ED compared to those with organic ED, although the former group had more sustained rigidity and a higher overall response rate. Side effects of nausea and yawning/stretching occurred more frequently with Melanotan II than with placebo, including a 15.3% incidence of severe nausea. The observation of increased sexual desire with Melanotan II is novel and warrants further investigation. Further studies to localize the site of action and receptor subtype mediating the action of Melanotan II may allow development of targeted receptor-specific melanocortin therapy for ED.

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Appendix

Open discussion following Dr Wessells' presentation

Dr Broderick: Did you do any stimulation studies to see if you could increase the onset of erection and increase the duration of erection?

Dr Wessells: We are currently conducting a study with a few men where we're collecting pharmacokinetic data. We're showing them videos and sexual stimulation, so, hopefully I'll have an answer for you on that.

Dr Broderick: Were your men doing any daytime napping at home?

Dr Wessells: They were instructed not to sleep, and a flaw of the study was that we should have kept the men in the clinic rather than sending them home.

Dr Broderick: Do you believe these men were desirous of sexual activity?

Dr Wessells: There were some men who felt an increase in desire. They were stimulated, but it's going to take more studies to sort that out. We're always looking at drugs and saying we don't want them to increase sexual desire, but there may come a time where we try and develop a class of drugs that will increase desire.

Dr Nehra: The RigiScan itself is somewhat stimulating and may promote more erectile activity.

Dr Wessells: All of them were also evaluated with placebo, but yes, there may have been some sexual stimulation from the RigiScan.

PAIN MEDICINE
SECTION EDITOR
MICHAEL J. COUSINS

Chronic Blockade of Melanocortin Receptors Alleviates Allodynia in Rats with Neuropathic Pain

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We investigated the involvement of the spinal cord melanocortin (MC) system in neuropathic pain. Because we recently demonstrated that MC receptor ligands acutely alter nociception in an animal model of neuropathic pain, in this study we tested whether chronic administration was also effective. We hypothesized that chronic blockade of the spinal MC system might decrease sensory abnormalities associated with this condition. The effects of the MC receptor antagonist SHU9119 (0.5 μg/d) and agonist MTII (0.1 μg/d) were evaluated in rats with a chronic constriction injury of the sciatic nerve. Drugs were continuously infused into the cisterna magna. Antinociceptive effects were measured with tests involving temperature (10°C or 47.5°C) or mechanical (von Frey) stimulation. The administration of MTII increased mechanical allodynia, whereas SHU9119 produced a profound cold and mechanical antiallodynia, altering responses to control levels. The antiallodynic effects of SHU9119 were very similar to those produced by the α_2-adrenergic agonist tizanidine (50 μg/d). The effects of SHU9119 and MTII are most likely mediated through the MC4 receptor, because this is the only MC-receptor subtype present in the spinal cord. We conclude that the chronic administration of MC4-receptor antagonists might provide a promising tool in the treatment of neuropathic pain.

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Neuropathic pain (pain after a lesion to the central or peripheral nervous system) remains one of the most difficult forms of pain to treat. Conventional treatment with the two major classes of analgesics, nonsteroidal antiinflammatory drugs and opioids, is seldom effective. Moreover, the wide variety of drugs currently used in the treatment of neuropathic pain, including tricyclic antidepressants, anticonvulsants, the systemic administration of local anesthetics, and *N*-methyl-D-aspartate receptor antagonists, do not often provide adequate pain relief (1).

Extensive research with experimental animal models has led to the discovery of an array of potential new drug targets (2). A possible target in the control of neuropathic pain that has received very little attention is the melanocortin (MC) system. Several lines of research have suggested an involvement of the MC system in nociception. Previous studies have demonstrated hyperalgesia in different tests of acute nociception after intracerebroventricular administration of the MCs α-melanocyte-stimulating hormone (α-MSH) and adrenocorticotropic hormone (3,4). In addition, the analgesic effects of morphine and β-endorphin are antagonized by these peptides (5).

At the spinal cord level, the existence of a functional MC system is suggested by the presence of the pro-opio-melanocortin-derived peptides adrenocorticotropic hormone and α-MSH and the MC4 receptor (6,7). It is interesting to note that these are all colocalized in the superficial dorsal horn, an area that is important in nociception. Taken together, these findings suggest an involvement of the spinal cord MC system in nociceptive transmission.

We investigated the spinal cord MC system as a new drug target for the control of neuropathic pain. We have shown that a chronic constriction injury (CCI) of the sciatic nerve (8), a condition that causes a syndrome similar to human neuropathic pain, induces an increase in ^{125}I-labeled [Nle4, D-Phe4]-a-MSH binding to the spinal cord, suggesting an increase in MC receptors. Furthermore, acute intrathecal administration of the MC receptor antagonist SHU9119 (directly into the cisterna magna) reduced cold and mechanical allodynia in CCI rats, whereas MC4-selective agonists had the opposite effect (9).

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0003-2999/01

Most drugs used in animal models of neuropathic pain are tested in an acute administration paradigm. Because it is a chronic form of pain, drugs that are suitable for chronic administration and provide long-lasting pain relief are interesting from a clinical point of view. In light of the antiallodynic effect of acutely administered SHU9119, in this study we have investigated the effects of this compound, as well as the MC receptor agonist MTII, on chronic administration.

The effects of these MC-receptor ligands were compared with those of the α_2-adrenergic agonist tizanidine, for which the antinociceptive and antiallodynic actions in experimental animals have been well documented (10,11).

Methods

All procedures in this study were performed according to the Ethical Guidelines of the International Association for the Study of Pain (12) and approved of by the Ethics Committee on Animal Experiments of the Utrecht University.

Forty-eight male Wistar rats weighing 350–400 g at the start of the study were used. They were socially housed in groups of two or three on sawdust bedding. The animals were kept on a 12:12-h light/dark cycle, with food and water available *ad libitum*.

Animals were randomly assigned to different treatment groups. CCI rats were treated with vehicle (n = 10), SHU9119 0.5 μg/d (n = 11), tizanidine hydrochloride 50 μg/d (n = 7), or MTII 0.1 μg/d (n = 10). Sham rats (n = 10) were treated with vehicle.

SHU9119 (cyclo-[Nle^4, Asp^5, D-Nal(2)^7, Lys^{10}]α-MSH-[4–10]) was synthesized by using 9-fluorenylmethoxycarbonyl-based solid phase synthesis as reported elsewhere (13) and purified by using reversed-phase preparative high-pressure liquid chromatography to a purity of ±90%, estimated after analysis by analytical high-pressure liquid chromatography at 215 nm. Molecular weight was confirmed by mass spectrometry performed on a Micromass Quattro (Micromass, Manchester, UK). Tizanidine hydrochloride was purchased from Novartis Pharma AG (Basel, Switzerland). MTII (melanotan-II or cyclo-[Nle^4, Asp^5, D-Phe^7,Lys^{10}] α-MSH-[4–10]) was purchased from Bachem Feinchemicalien (Bubendorf, Switzerland).

Drugs were dissolved in saline and continuously administered into the cisterna magna via an Alzet osmotic minipump (type 2002; Charles River, Someren, the Netherlands; pump speed 0.5 μL/h for 14 days).

Before surgery, all animals were anesthetized with a single subcutaneous injection of Hypnorm (Janssen Pharmaceutical Ltd., Beerse, Belgium) containing 0.315 mg/mL fentanyl citrate and 10 mg/mL fluanisone (a butyrophenone), at a dose of 0.3 mL/kg body weight. A CCI was produced by placing four loose ligatures of 4-O chromic catgut (Ethicon Inc., Johnson & Johnson, Somerville, NJ) around the nerve as previously described by Bennett and Xie (8). Subsequently the incision was closed with silk sutures, and the animals were allowed a 2- to 3-day recovery period. For the sham condition, the same procedure was performed except for placement of the ligatures.

Two weeks after the initial surgery, a steel cisterna magna cannula (20 × 0.4 mm) was placed as described by Lankhorst et al. (14). An Alzet osmotic minipump (type 2002; pumping rate, 0.5 μL/h; duration, 14 days), filled with the appropriate solution, was implanted into the right flank and connected to the cannula by subcutaneously tunneled polyethylene tubing (PE 25). The incision in the flank was closed with silk sutures. Because the tube connecting the minipump to the cannula was filled with saline and as a consequence of the length of the tube and pump speed, the drugs were delivered into the cerebrospinal fluid (CSF) starting 3 days after implantation of the pumps. This allowed the animals a 3-day recovery period before testing was initiated. The day on which the drugs were delivered into the CSF is referred to as "Treatment Day 1."

Withdrawal latency to a temperature stimulus was measured by immersing the hind paws on each side into a 10°C or 47.5°C water bath. Upon immersion of the paw, an electronic circuit including a timer was closed. Withdrawal of the paw resulted in a discontinuation of the circuit, which stopped the timer, thus allowing a precise registration of the withdrawal latency time. Cutoff time was set at 10 s to avoid skin damage. The interval of time between consecutive tests was at least 10 min to allow restoration of the original foot temperature.

Before testing, each rat was placed in a plastic testing box with a metal mesh floor and allowed to acclimatize to this environment. Mechanical allodynia was determined by measuring the paw withdrawal threshold after probing of the foot plantar surface with a series of calibrated von Frey filaments (Stoelting, Wood Dale, IL), ranging from 1.08 to 21.09 g. Probing was performed only if the hind paw was in contact with the mesh floor, to correct for paw lifting in response to spontaneous pain. Filaments were applied to the midplantar surface of both feet through the mesh floor until the filament bent and were kept in this position for 6–8 s (15). The smallest force that elicited a foot withdrawal response was considered the threshold stimulus.

Temperature and mechanical stimulation tests were performed the day before the beginning of pharmacologic treatment ("baseline value") and at 2- to 3-day intervals thereafter ("Treatment Days 1–10"). Throughout the experiment, body weight was monitored at regular intervals.

Data are plotted starting from the day before the onset of treatment (baseline) through Treatment Day 10. Withdrawal latencies are expressed as mean ± SEM. Withdrawal thresholds to von Frey stimulation are expressed as median and 25th to 75th percentile range, with values plotted on a logarithmic scale.

Differences in body weight between treatment groups were compared by using a repeated-measures analysis of variance. All other data were analyzed with nonparametric tests. To obtain a linear scale of perceived force in the mechanical stimulation test, withdrawal thresholds were converted to the log of the actual bending force of the filament. Statistical analysis was performed on the transformed data. Differences in baseline values were analyzed with a Mann-Whitney *U*-test. Overall group differences in mechanical and temperature stimulation tests were analyzed by a Kruskal-Wallis test. The effects of the different drugs were compared with the vehicle-treated CCI group and, in case of a significant difference, with the Sham group by using a Mann-Whitney *U*-test with Bonferroni's correction. In the mechanical stimulation test, for the CCI group treated with MTII, thresholds on Treatment Days 1 and 3 were compared with baseline values by using a paired Student's *t*-test. Results were considered significant when $P < 0.05$. For the cold and mechanical stimulation tests, the effect of SHU9119 or tizanidine was quantified as the percentage of maximum possible effect (%MPE), by using the following formula:

$$\%\text{MPE} = 100 \times \frac{(\text{postdrug value} - \text{baseline value})}{(\text{cutoff value} - \text{baseline value})}$$

Results

At the end of the experiments, proper placement of cannulas and connection to the minipumps was checked. Pump reservoirs were checked to control accurate drug delivery. All reservoirs were empty. One animal was excluded because of improper connection of the pump, and one animal died during anesthesia. Both were CCI animals. There were no significant differences in body weight between treatment groups at any time point (data not shown).

All CCI groups developed a cold allodynia of the ligated hind paw, as indicated by a significant reduction in withdrawal latency upon immersion in a 10°C water bath (mean predrug value ± SEM for all CCI groups, 5.5 ± 0.7 s). In the Sham group, none of the animals showed signs of cold allodynia (mean withdrawal latency ± SEM, 9.5 ± 0.3 s). The cutoff latency for the test was 10 s. No signs of cold allodynia developed in the contralateral hind paw of either Sham or CCI animals.

There were no significant differences in baseline responses to heat (47.5°C) stimulation between Sham and CCI animals on either side.

CCI animals displayed a tactile allodynia on the ligated side, as shown by a significant decrease in withdrawal threshold to von Frey stimulation (predrug value ± SEM for all groups of 8.5 [6.2–8.5] g) (median [25th –75th percentile]). Sham animals failed to respond to any filament up to the maximum of 21.09 g, as was the case for the contralateral hind paw of CCI animals. These data are summarized in Figure 1.

As shown in Figure 2A, treatment with SHU9119 (500 ng/d) significantly prolonged withdrawal latency of the ligated hind paw to a cold stimulus as compared with the vehicle CCI group ($P < 0.05$ on Treatment Days 1 and 3), restoring latencies to near cutoff values (nonsignificant versus Sham). %MPE was 86.9% ± 8.1% and 90.7% ± 9.3%, respectively.

This effect of SHU9119 was similar to that of tizanidine 50 μg/d ($P < 0.05$ versus the vehicle CCI group on Treatment Days 1 and 3) (Fig. 2A); %MPE was 97.3% ± 2.7% and 100% ± 0%, respectively. On Treatment Day 5, withdrawal latencies decreased again in both SHU9119- and tizanidine-treated groups and did not significantly differ from the vehicle CCI group throughout the rest of the testing period.

Treatment with MTII (100 ng/d) resulted in a transient, nonsignificant decrease in withdrawal latencies of the ligated hind paw (Fig. 2A).

All treatments were ineffective in causing any significant changes in withdrawal latencies to the cold stimulus on the contralateral side. Also, there were no significant differences in withdrawal latency to a heat stimulus between the vehicle-treated CCI group and any of the other treatment groups on either side (data not shown).

As shown in Figure 2B, treatment with SHU9119 (500 ng/d) increased the withdrawal threshold on the ligated side up to 17.0 [13.2–20.9] and 20.9 [13.2–20.9] g (median [25th –75th percentile]) on Treatment Days 1 and 3; % MPE was 73.0% [26.4%–100%] and 100% [46.6%–100%], respectively. These thresholds were significantly higher than those in the vehicle CCI group ($P < 0.05$) and did not significantly differ from the maximum threshold observed in the Sham-Operated group.

The administration of tizanidine 50 μg/d resulted in a similar increase in threshold. On Treatment Days 1 and 3, thresholds were 13.2 [13.2–13.2] and 13.2 [13.2–20.9] g, respectively ($P < 0.05$ versus vehicle CCI group), with corresponding %MPE of 46.6% [24.3%–52.2%] and 46.6% [37.7%–100%] (median [25th–75th percentile]). Only on Treatment Day 3 did this threshold not significantly differ from the threshold of the Sham-Operated group.

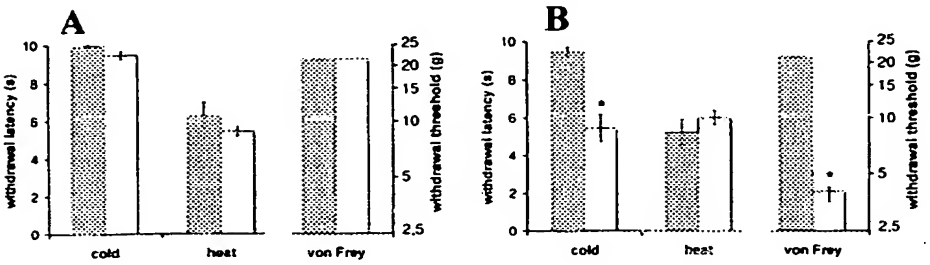

Figure 1. Baseline withdrawal latencies to cold (10°C) and heat (47.5°C) stimulation and baseline withdrawal thresholds to von Frey stimulation in Sham (gray bars) and Chronic Constriction Injury (CCI) (open bars) rats. Measurements were taken on the unoperated (A) and experimental (B) hind paw. Withdrawal latencies are presented as mean ± SEM and withdrawal thresholds as median and 25th-75th percentile range (logarithmic scale) of 10 (Sham) or 36 (CCI) rats. $^*P < 0.05$ versus Sham.

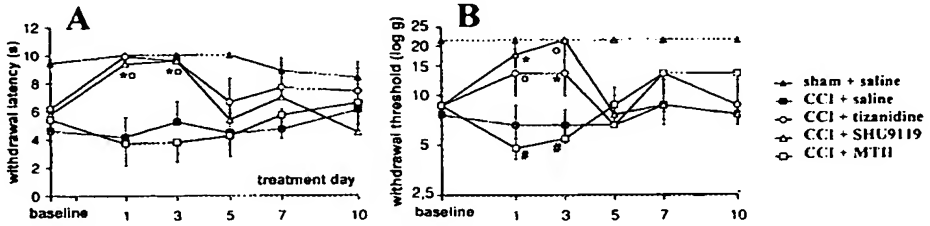

Figure 2. The effect of SHU9119 (500 ng/d), MTII (100 ng/d), and tizanidine (50 μg/d) on withdrawal latencies to cold (10°C) stimulation (A) and withdrawal thresholds to mechanical stimulation (B) in rats with a chronic constriction injury (CCI). Drugs were continuously administered into the cisterna magna by an osmotic minipump. Data are plotted from the day before the beginning of treatment (baseline) to Treatment Day 10. Withdrawal latencies are presented as mean ± SEM and withdrawal thresholds as median and 25th-75th percentile range (logarithmic scale) of 10 (sham, CCI + vehicle, and SHU9119), 9 (MTII), or 7 (tizanidine) rats each. $^*P < 0.05$, SHU9119 versus vehicle; $^\circ P < 0.05$, tizanidine versus vehicle; $\#P < 0.05$ versus baseline.

On Treatment Day 5, thresholds in both the SHU9119 and tizanidine-treated groups decreased again and were not significantly different from the vehicle CCI group for the remainder of the testing period.

Treatment with MTII 100 ng/d produced a decrease in withdrawal thresholds on Treatment Days 1 and 3 (median [25th –75th percentile] of 4.7 [4.1–5.4] and 5.4 [5.0–5.4]), respectively (Fig. 2B). When compared with the vehicle CCI group, this decrease was not significant because of a slightly higher baseline value in the MTII-treated group. However, within the MTII-treated group, withdrawal thresholds on Treatment Days 1 and 3 were significantly lower than the baseline value.

None of the treatments caused any significant changes in withdrawal threshold on the contralateral side (data not shown).

Discussion

Here we demonstrate that the MC-receptor antagonist SHU9119, when infused chronically into the cisterna magna, reduces cold and mechanical allodynia in rats with CCI. Infusion of MTII, an MC-receptor agonist, into the cisterna magna induces an increased sensitivity to mechanical stimulation. Because the MC4 receptor is the only MC-receptor subtype present in the spinal cord (16), it is most likely that the observed effects of MTII and SHU9119 are mediated through this receptor, as we have previously suggested (9).

The mechanism through which SHU9119 alleviates allodynia remains to be elucidated. Possibly the endogenous MC-receptor agonist α-MSH, released in the dorsal horn (6), exerts a tonic influence on nociception. Consistent with this view is the induction of hyperalgesia upon intracerebroventricular administration of MC-receptor agonists (3,4) and the observed upregulation of spinal cord MC receptors in rats with CCI (9); this could contribute to the increased sensitivity associated with the lesion. We hypothesize that the antiallodynic effects of SHU9119 are caused by a blockade of the endogenous α-MSH tonus.

MCs modulate a variety of body functions, including fever, immunity, and body weight homeostasis (17). Body weight homeostasis has received much attention because the MC4 receptor plays a role in disorders of energy balance, such as obesity (18). In rats, MTII and SHU9119 respectively inhibit and stimulate food intake when administered intracerebroventricularly (19). With the use of MCs as possible analgesic drugs, changes in body weight are unwanted side effects. Therefore, in this study, drugs were continuously infused into the cisterna magna, downstream of the cerebroventricular system. The osmotic minipumps we used have a very slow infusion speed (0.5

μL/h), thus allowing a very gradual release of drugs without creating pressure in the direction of the ventricular system. The continuity and speed of drug delivery by the minipumps have been verified earlier in our laboratory (unpublished results). Because at the end of the study, pump reservoirs were empty, it is very unlikely that there was backflow from CSF into the pumps. Thus, in this way drugs were delivered directly in the CSF surrounding the spinal cord, where their proposed site of action, the spinal MC4 receptor, is located. The doses of SHU9119 and MTII we used here (0.5 and 0.1 μg/d, respectively) have been shown in rats to readily affect body weight when administered intracerebroventricularly (19). Here we show that the antiallodynia induced by the chronic administration of the MC-receptor antagonist SHU9119 into the cisterna magna is not accompanied by any changes in body weight, further confirming a spinal site of action.

The magnitude of the antiallodynic effects we find with SHU9119 are comparable to those of tizanidine and were so large that responses were normalized to control (sham) levels. Moreover, the cold antiallodynia corresponds well with that described by Leiphart et al. (10) (more than an 80% decrease in paw withdrawal after 50 μg tizanidine intrathecally). In contrast, they describe a %MPE of only 19% in the paw pinch test, whereas we observed a %MPE of up to 46% in the von Frey test with tizanidine 50 μg/d. This might be explained by the fact that the paw pinch test measures pressure hyperalgesia, whereas the von Frey stimulation that we used measures mechanical allodynia. It is, however, difficult to make full comparisons between tizanidine and SHU9119, because only single doses were tested, and the mechanisms of action (involving the α-adrenoreceptors for tizanidine and the MC receptors for SHU9119) were clearly distinct.

In contrast to other groups (8,20), we found no differences in heat sensitivity between CCI and control rats. This discrepancy might result from genetic differences between various rat strains, leading to variations in the predisposition for the development of neuropathic conditions (21) or in sensitivity to noxious stimuli (22). In this study, none of the drugs tested induced changes in the CCI animals' responses to heat. Also, we have previously demonstrated that injecting a large dose of MTII (500 ng) or SHU9119 (1.5 μg) into the cisterna magna does not affect pain perception in control rats (9). Taken together, these data suggest that both tizanidine and the MCs specifically affect the sensory abnormalities associated with the neuropathic pain state without affecting normal pain perception. Our data confirm previous reports of this selectivity of tizanidine effects in neuropathic pain (10,11).

Despite its potent antinociceptive actions in experimental animals, clinical trials performed with tizanidine had less promising results. In patients with trigeminal neuralgia, the efficacy of tizanidine was inferior to that of carbamazepine (23), and there was a rapid recurrence of painful attacks during tizanidine treatment (24).

Levy et al. (11) have reported that upon chronic intrathecal infusion of tizanidine, after several days rats became tolerant to its analgesic effects. In this study, we observed a similar time course of the effects of tizanidine. Surprisingly, we found that MTII and SHU9119 also had only temporarily effects. Possibly the organism quickly adapts to both a lack of tonic α-MSH and a continuous overstimulation of MC receptors, as occur with chronic infusion of SHU9119 and MTII, respectively. These adaptations or development of tolerance might be suppressed by using other dosages of drugs or different drug administration regimens, such as repeated injections at various intervals. Future experiments using these strategies will be helpful in further addressing this question.

However, this rapid decline of effects of the MCs should not exclude MC antagonists from further consideration in the treatment of neuropathic pain in humans, because differences in the speed of the development of tolerance between rats and humans do occur. For instance, tolerance to intrathecal morphine develops quickly in rats in several tests of acute nociception (25), whereas in humans, morphine tolerance can take several months to develop, and morphine can provide long-lasting adequate pain relief in cancer pain (26) and nonmalignant pain (27).

In summary, we demonstrate that chronic intrathecal infusion of the MC-receptor antagonist SHU9119 has profound antiallodynic effects in rats with a CCI of the sciatic nerve. We suggest that these effects are mediated through the spinal cord MC4 receptor. SHU9119 seems to be specifically effective in altering the sensory abnormalities associated with the neuropathic pain state, without affecting normal pain perception. Therefore, we suggest that selective MC4 antagonists might be of value in the treatment of neuropathic pain.

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MINIREVIEW

MELANOCORTINS AND OPIATE ADDICTION

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Summary

Adrenocorticotropic hormone (ACTH) and α-melanocyte stimulating hormone (α-MSH) are centrally acting melanocortin peptides with numerous reported functions, including induction of excessive grooming and antipyresis, among others. Also reported is a role for melanocortins in aspects of opiate action. Although early work examined the effects of ACTH and MSH on opiate-induced behaviors, further progress has been limited. Recently, however, advances in the identification and characterization of melanocortin receptor (MC-R) subtypes have provided novel tools with which to study interactions between melanocortins and addiction. The present review discusses the effects of ACTH and MSH on opiate-induced behaviors and relates these findings to more recent reports on the regulation of melanocortin systems by exogenous opiates. Emerging from these data is the possibility that melanocortin receptor activation, specifically at the MC4-R subtype, may act to antagonize certain properties of exogenous opiates, including perhaps addiction.

Key Words: POMC, ACTH, MSH, opiates, addiction

Introduction: Neurobiology of Melanocortins

The melanocortin peptides, which include ACTH, α-MSH, β-MSH, and γ-MSH, are derived from the proteolytic processing of the pro-opiomelanocortin (POMC) precursor protein (1). The POMC gene is expressed in many peripheral tissues (2), but is most highly expressed in the anterior and intermediate pituitary lobes (1). In the central nervous system, POMC expression is confined largely to the arcuate nucleus of the hypothalamus (3) and nucleus tractus solitarius of the medulla (4, 5). From these two discrete sources, melanocortinergic fibers project widely throughout the brain (3, 6).

Following the discovery of specific binding sites for melanocortins in the central nervous system (7), the first MC-Rs were cloned and characterized (8). To date, five MC-R subtypes have been identified (8-21). Each receptor subtype activates adenylyl cyclase, and several may activate phospholipase C as well (22, 23). RNA encoding four of the receptor subtypes has been reported to be expressed in brain (Table 1). MC1-R mRNA has been detected only in a very small population of neurons within the periaqueductal gray (PAG) (24). MC5-R mRNA has also been shown to be expressed at extremely low levels in several brain regions (18). MC3-R mRNA is present in relative abundance in a select number of areas including the hypothalamus, septum, ventral tegmentum, and raphe nucleus (11). By far, the most abundant and most widely distributed melanocortin receptor subtype in the brain is MC4-R (Table 1) (16, 21).

Functions for the melanocortin receptors and peptides in the central nervous system are quite disparate. One of the best characterized effects of α-MSH or ACTH is the induction of excessive grooming behavior in rats following intracerebroventricular administration (25) or direct infusion into the ventral tegmentum or substantia nigra (26). ACTH and MSH are also known to be potent antipyretic (27, 28) and antiinflammatory (29-32) agents. In addition, they have neurotrophic effects in the periphery (33) and perhaps in the brain (34-37). Finally, melanocortins are purported to facilitate learning (38-40).

Interaction between Melanocortins and Opiates

A number of studies have suggested that melanocortins interfere with or antagonize the actions of exogenous opiates. Several lines of evidence support this hypothesis. Electrophysiological studies have indicated that melanocortins block morphine-induced depression of evoked potentials in the isolated frog spinal cord (41) as well as in the lumbar ventral root of decerebrate-spinal cats (42). In addition, prior to the discovery of melanocortin receptors, pharmacological studies indicated

TABLE 1

Regional Distribution of MC-R mRNA Expression in Rat Brain

	MC1-R	MC2-R	MC3-R	MC4-R	MC5-R
cortex	-	-	-	+	-/+
hippocampus	-	-	+	+	-/+
olfactory bulb	-	-	-	++	-/+
neostriatum	-	-	-/+	++++	-/+
nucleus accumbens	-	-	-/+	++++++	-/+
hypothalamus	-	-	+++	+++	-/+
septum	-	-	++	+++++++	nd
periaqueductal gray	-/+	-	+	++++	-/+
ventral tegmentum	-	-	++	++	nd
substantia nigra	-	-	nd	+	-/+
cerebellum	-	-	-	+	-/+

Semiquantitative estimates based on references 11, 16, 18, and 21. Increasing numbers of +'s denote greater enrichment of mRNA expression; nd (not determined), - (not detected), -/+ (at limits of detection).

that ACTH-like peptides bind with micromolar affinities to opiate receptors and act as partial opiate antagonists (43-46). Moreover, functional studies have demonstrated that melanocortins reduce the analgesic effect of morphine (47-49), although reports exist to the contrary (50, 51).

Most importantly for the present review, behavioral investigations have demonstrated that *in vivo* administration of melanocortins antagonizes the addictive properties of opiates. For example, it has been shown that melanocortins attenuate acquisition of heroin self-administration (52). In addition, they inhibit the development of both tolerance and physical dependence to opiates (49, 53). Finally, melanocortins can induce an opiate withdrawal-like syndrome in drug-naive animals (52, 54). Arising from the behavioral literature, then, is the possibility that melanocortin administration may impede several actions of exogenous opiates, including the development of addiction.

Opiate Regulation of Melanocortin Systems

The above discussion raises the question of whether exogenous opiates must somehow overcome antagonism by endogenous melanocortin systems in order for opiate tolerance and dependence to develop. It is possible that opiates achieve this by dampening MC-R activation, either by decreasing neuronal sources of melanocortin peptides and/or by decreasing receptor levels. Recent molecular biological evidence supports both of these possibilities. Several groups have reported that chronic administration of morphine down-regulates the expression of POMC mRNA in the hypothalamus, as measured by Northern blot analysis (55, 56), RNase Protection Assay (RPA) (57), and *in situ* hybridization (58). In these studies, it was also demonstrated that chronic morphine administration decreases the release and/or synthesis of β-endorphin and another POMC derived peptide, corticotropin-like intermediate lobe peptide (56, 57). Because melanocortins arise from this same POMC precursor, it can be inferred from these data that morphine also decreases the bioavailability of ACTH and MSH.

Most recently, our own work has shown that in a drug administration paradigm known to produce opiate tolerance and dependence, as well as withdrawal upon removal of drug (59), morphine down-regulates MC4-R mRNA expression (21). This particular subtype was studied for several reasons. First, we have shown that it is enriched in regions which may mediate aspects of drug addiction (Table 1) (21). Second, structure-activity relationships have correlated the relative ability of various melanocortin peptides to block opiate tolerance with their ability to activate MC4-R *in vitro* (46, 60). Strikingly, the down-regulation of this receptor by opiates is limited specifically to brain regions implicated in the etiology of addiction. In the nucleus accumbens, which is a principal site for the reinforcing properties of opiates and other drugs of abuse (61-63), morphine decreases MC4-R mRNA expression after only one to three days of treatment (Fig. 1A). Interestingly, this effect reverses after 5 days of morphine administration. In the PAG, an anatomical region important for the development of physical dependence to morphine (64), MC4-R mRNA was found to decrease after five days of drug treatment (Fig. 1A). Finally, in the neostriatum, which may play a role in mediating the psychomotor activating effects (65) and even the reinforcing properties (66) of opiates, five days of morphine treatment also decreases MC4-R mRNA levels (Fig 1A). In this region, the reduction in receptor mRNA is accompanied by a concomitant decrease in melanocortin receptor levels, as determined by radioligand binding and autoradiography (Fig. 1B). It is believed that this reduction in receptor binding represents a decrease in MC4-R, as the binding pharmacology of MC-Rs in this brain region is consistent with that of MC4-R but not with that of MC3-R, the other principal MC-R subtype expressed in this region (67). In contrast, morphine does not alter MC4-R mRNA levels in several other brain areas including the olfactory bulb, hypothalamus, frontal cortex, and ventral tegmentum/substantia nigra. Addition-

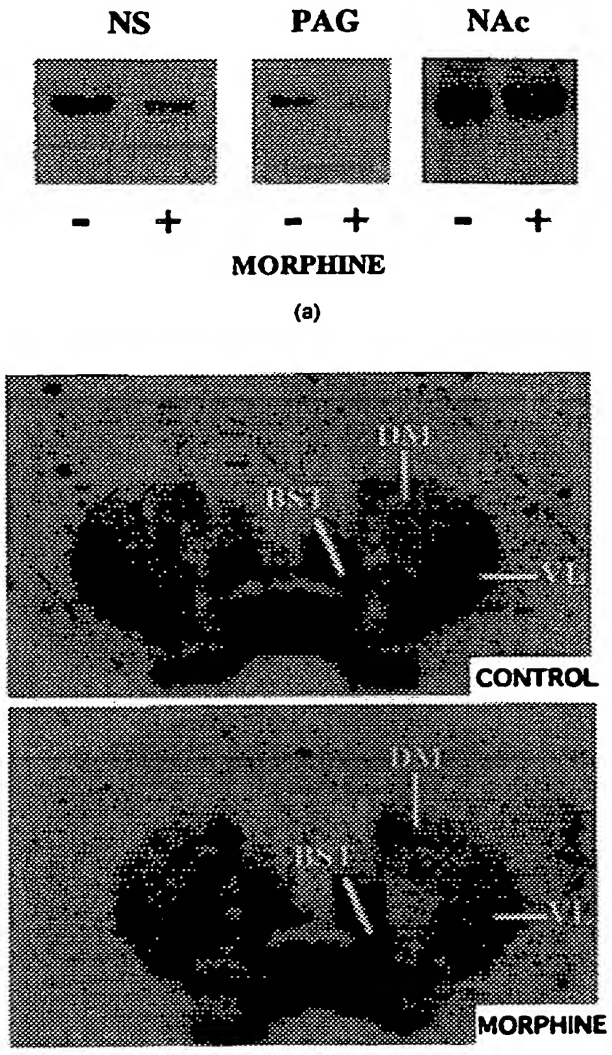

FIG. 1

Opiate regulation of MC4-R. Panel a, Polyacrylamide gel electrophoresis of Rnase protected MC4-R mRNA in neostriatum (NS), periaqueductal gray (PAG), and nucleus accumbens (NAc) following chronic morphine administration (3 days, NAc; 5 days PAG, NS). Panel b, ^{125}I -NDP MSH receptor ligand binding autoradiography at the level of the anterior commissure in control and 5 day morphine treated rats. Binding in the ventrolateral striatum (VL), but not in the adjacent dorsomedial striatum (DM) and bed nucleus of the stria terminalis (BST), was significantly decreased after morphine treatment. See Alvaro et al (1996) for further results and methodological details.

ally, although other MC-R subtypes have been reported to be expressed in the nucleus accumbens, neostriatum, and PAG (11, 18, 24), we have found that mRNA encoding these receptors are nearly undetectable or are unregulated by morphine (21).

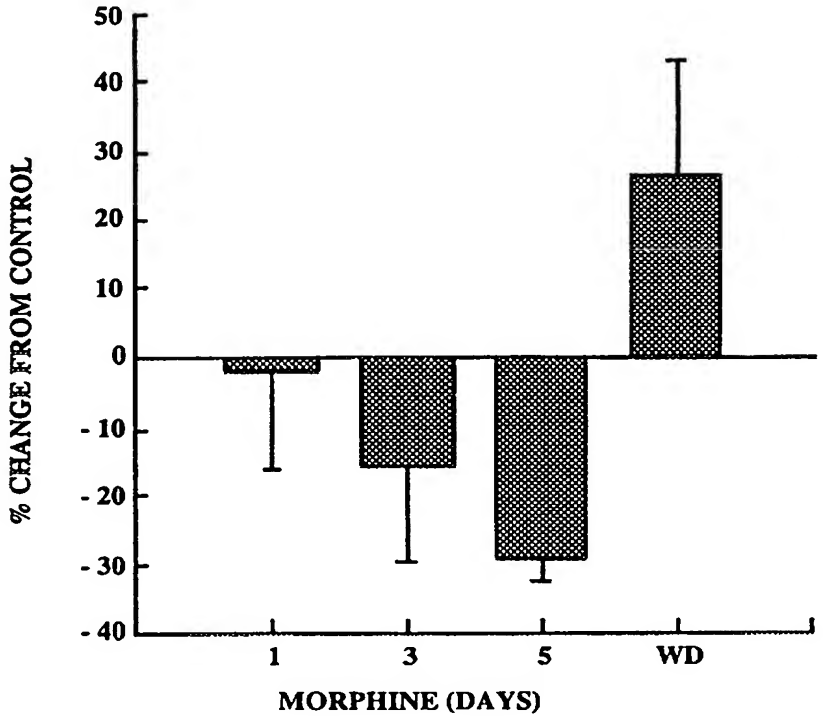

FIG. 2

MC4-R mRNA levels in neostriatum following 1, 3, or 5 days morphine administration as well as 6 hr after naloxone-precipitated withdrawal (WD) in 5 day treated animals. Levels of MC4-R mRNA were determined by RNase protection assay, and autoradiograms of acrylamide gel results were quantified by laser densitometry. The results are expressed as percent change from control.

Taken together, the decreases in POMC expression, bioavalability of POMC-derived peptides, and MC4-R expression lend credence to the hypothesis that chronic opiate treatment does decrease melanocortin receptor activation. It is interesting to note that when withdrawal is induced by naloxone administration in rats chronically treated with morphine, the previously described decreases in MC4-R mRNA in the neostriatum (Fig. 2) and PAG (not shown) reverse within six hours. This finding, if it signals the rapid return of MC4-R activation to basal levels, parallels the discovery that melanocortin administration into the PAG of drug-naive animals induces an opiate withdrawal-like effect (52, 54).

Mechanisms of Melanocortin Antagonism of Opiates

The mechanisms through which melanocortins antagonize the effects of exogenous opiates are unknown. Based on several early reports (43-46), it is possible that MSH and ACTH are very low affinity opiate receptor antagonists. Alternatively, because MC-Rs and opiate receptors are enriched in several of the same brain regions including the neostriatum and nucleus accumbens (68), it is also possible that these receptor types are co-expressed in the same neurons. If this is the case, functional antagonism could occur intracellularly at the second messenger level. As stated previously, MC-Rs positively couple to adenylyl cyclase to stimulate adenosine 3', 5'-cyclic monophosphate (cAMP) production. Opiate receptors, on the other hand, inhibit adenylyl cyclase activation (69) via coupling to the G-proteins G_i and G_o (70). The opposite effects on cAMP pro-

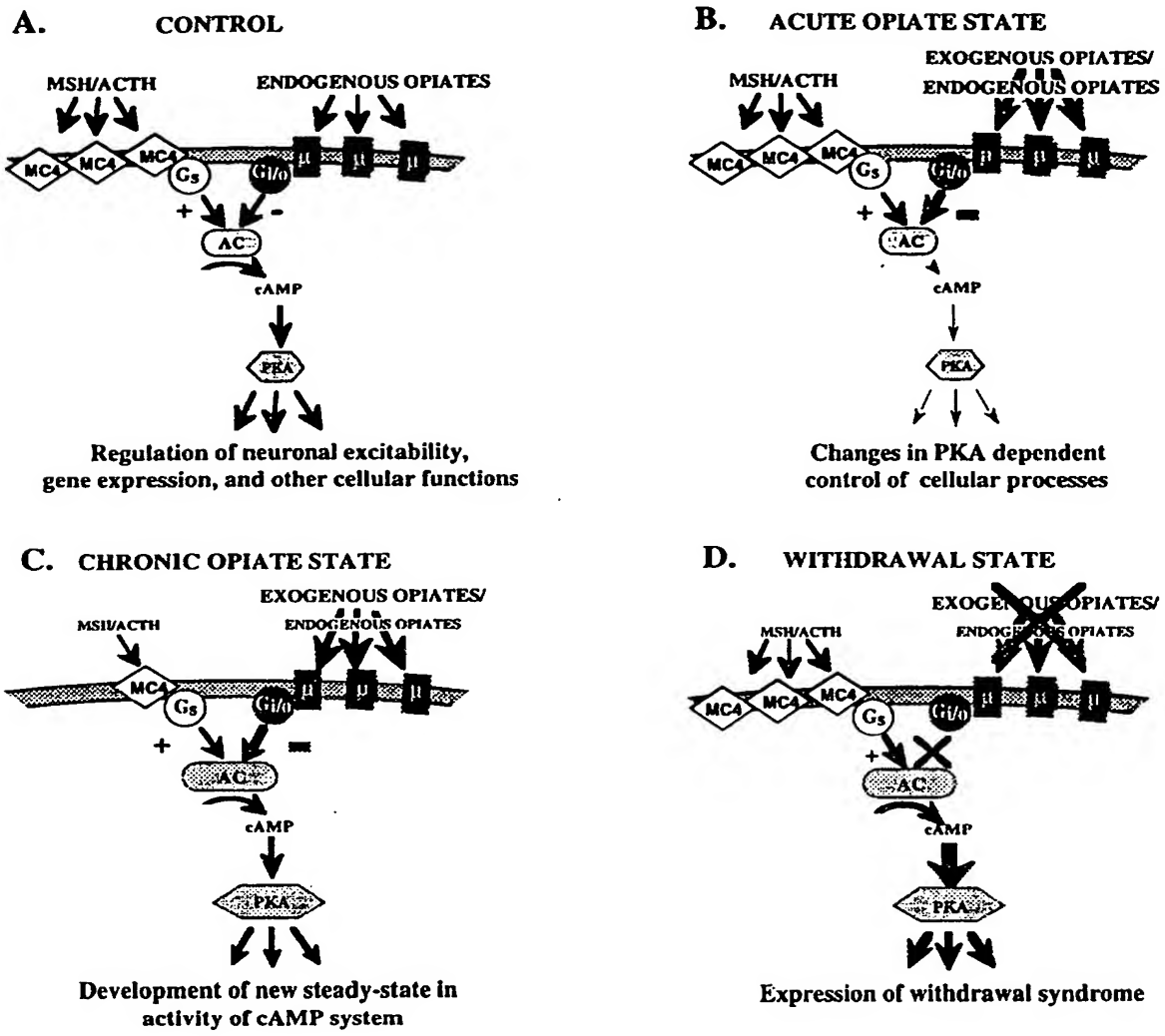

FIG. 3

Model of melanocortin and opiate interactions. Panel A. MC4-Rs activate while μ-opiate receptors inhibit adenylyl cyclase (AC) to regulate cAMP production and protein kinase A (PKA) activation. Panel B, exogenous opiate administration maximally stimulates opiate receptors to inhibit AC activation and PKA activity. Panel C, melanocortins and MC4-R are down-regulated by chronic exogenous opiate treatment, whereas opiate receptors remain maximally activated. To compensate for chronically decreased PKA activity, neurons in some brain regions may adapt by up-regulating levels of AC and PKA (71). Panel D, induction of withdrawal by removal of exogenous opiates and/or administration of opiate receptor antagonists. Opiate inhibition of AC is derepressed, and AC activity increases. As part of the withdrawal response, MC4-R expression rapidly returns to basal levels to further increase AC and PKA activity.

duction and subsequent protein kinase A (PKA) activation could be one means through which melanocortins antagonize the effects of opiate administration (Fig. 3A).

Also shown in Figure 3 is a model of the biochemical adaptations which may contribute to the development of dependence and the expression of withdrawal. Following acute opiate administration, activation of opiate receptors inhibits adenylyl cyclase activity and decreases activation of PKA (Fig. 3B). Repeated opiate treatment down-regulates melanocortins and MC4-R. The resulting persistent inhibition of the cAMP system by chronic opiate administration leads to a compensatory increase in adenylyl cyclase and PKA protein levels (Fig. 3C) in several brain regions including the nucleus accumbens, as described by others (71). Thus, in the opiate dependent state, functional inhibition of cAMP dependent signal transduction is countered by up-regulation of several components of the second messenger system. During the induction of withdrawal, in which opiate receptors are no longer activated, adenylyl cyclase is released from opiate inhibition. In addition, MC4-R rapidly returns to basal levels. The end result of MC-R activation would be an induction of the up-regulated cAMP system and a subsequent massive stimulation of PKA (Fig. 3D), which in turn might contribute to the expression of the behavioral manifestations of withdrawal.

Future Studies of Melanocortins in Drug Abuse

Whether the noted regulation of melanocortin systems by opiates directly contributes to the development of addiction and the expression of withdrawal remains to be demonstrated. At the receptor level, several approaches are available to address this question. Infusion of specific receptor antagonists or antisense oligonucleotides into discrete brain regions can be used to mimic MC4-R down-regulation. If this receptor does play a role in opiate addiction, such perturbations of MC4-R activation might be expected to enhance the development of tolerance and dependence or accelerate the acquisition of heroin self-administration. Also, these disruptions of MC4-R function might alleviate withdrawal symptoms. Conversely, infusion of MC4-R-specific agonists might be expected to block the development of the addicted state and increase the severity of withdrawal. In a similar way, it may soon be feasible to examine the effects of opiates in transgenic MC4-R knockout mice and mice with inducible MC4-R over-expression.

In addition to pursuing the above avenues of research, it is equally important to study the role of melanocortins in behavioral responses to other drugs of abuse. Both opiate and cocaine administration have previously been shown to regulate several of the same signal transduction proteins in brain reward regions (72), and with this in mind, we have initiated studies into the regulatory effects of cocaine on POMC and MC-Rs. Our preliminary results indicate that chronic cocaine administration does regulate MC4-R mRNA in several brain regions including the neostriatum (73). The robust regulation of MC4-R by cocaine in our initial experiments raises the novel possibility that melanocortin systems are key targets of cocaine action in the brain. With this impetus, it is believed that our ongoing work with opiates, cocaine, and other drugs will further define the exact nature of the relationship between melanocortins and drugs of abuse and may eventually lead to the discovery of new therapeutics for the treatment of addiction.

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